

# Fungal plant pathogens on inoculated maize leaves in a simulated soil warming experiment

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## ABSTRACT

Climate change will affect the survival of plant pathogens and the decomposition of crop residues on temperate arable soils. Information on the survival of fungal plant pathogens is important for assessing the subsequent infection risk of susceptible agricultural crops. Therefore, a soil warming experiment was performed to examine the effects of rising winter soil temperatures on the inoculum load of fungal plant pathogens (*Fusarium culmorum*, *F. graminearum*, *Rhizoctonia solani*) and the decomposition of infested maize leaves under field conditions. Using heating cables, an arable soil was subjected to temperature treatments simulating medium (+1.3 °C up to the year 2050) and long-term (+2.7 °C up to the year 2100) climate warming scenarios. Litterbags filled with pathogen-inoculated and non-inoculated maize leaves were placed on top of the soil. Soil microbial biomass below the litterbags, maize leaf decomposition as well as microbial colonisation of leaves were measured after 152 days. Pathogen load was estimated by DNA, total saprotrophic biomass by fungal glucosamine and bacterial muramic acid. Rising soil temperatures increased decomposition of pathogen-infested, but not that of non-inoculated maize leaves, without correlation to fungal and bacterial biomass. *F. culmorum* DNA produced the largest increase in DNA on maize leaves, but did not significantly respond to soil temperatures. In contrast, the increase in *F. graminearum* DNA was considerably lower, but revealed a significant positive response to rising soil temperatures. DNA from mycelia and sclerotia of *R. solani* strongly decreased. Rising winter soil temperatures will most likely cause shifts within the plant residue colonizing fungal community, especially between different *Fusarium* species.

## 1. Introduction

In wheat and other cereal crops, *Fusarium* species cause several diseases such as seedling blight, foot rot, *Fusarium* head blight, ear rot of maize and crown rot in wheat and barley (Doohan et al., 2003; Xu and Nicholson, 2009). *Fusarium culmorum* WG Smith and *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schw.) Petch) belong to the most important pathogens in crops worldwide, leading to high yield losses and to contamination of the grain with mycotoxins, such as deoxynivalenol and zearalenone (Basler, 2016; Pasquali et al., 2016; Popiel et al., 2008). The soil-borne fungus *Rhizoctonia solani* Kühn (teleomorph *Thanatephorus cucumeris* (Frank) Donk) with the anastomosis group (AG) *R. solani* AG 2-2 IIIB is responsible for diseases in sugar beet (*Beta vulgaris* L.), decreasing yields by up to 50% in parts of Europe, Japan and the United States (Kiewnick et al., 2001; Kühn et al., 2009).

Crop residues on the soil surface, in particular large amounts of maize debris, serve as substrate for these fungi; they represent a major source of inoculum for crop plant infection in the next season (Maiorano et al., 2008; Palumbo et al., 2008). However, fungal survival and inoculum production on crop residues is limited by residue decomposition. In contrast to plant residues buried in soil after ploughing, residues on the soil surface provide nutrients and serve as a substrate for a longer period, allowing fungal plant pathogens to survive for several years (Pereyra et al., 2004; Vogelgsang et al., 2011). Climate change may have a strong impact on pathogen population dynamics and decomposition processes of crop residues on temperate arable soils, as they remain close to freezing point throughout the winter (Henry, 2008). Here, temperature and water are key conditions influencing saprophytic growth and survival of fungal pathogens and the severity of diseases they cause (Doohan et al., 2003). In Europe and especially in Germany, climate change has led to increased mean air temperatures

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over the last 50 years (Haberlandt et al., 2010) and trend analysis has shown an increase in the mean annual temperature of about 0.8 to 1.1 °C from 1901 to 2000, accompanied by a marked increase in winter precipitation (Schönwiese and Janoschitz, 2008).

Knowledge concerning the effects of changing winter climate temperatures on infestation and colonisation of crop residues and the survival of fungal plant pathogens is important for assessing the subsequent infection risk of susceptible agricultural crops (Juroszek and von Tiedemann, 2011, 2013a,b; Launay et al., 2014). Currently, there is limited information available (Siebold and von Tiedemann, 2012a). Litterbag experiments are often used to investigate decomposition of plant residues (Knacker et al., 2003; Jacobs et al., 2011). They provide a useful tool for studying the effects of soil temperatures on the survival of fungal plant pathogens on crop debris under field conditions. Colonisation of decomposing plant material by fungi and bacteria can be specifically quantified by the analysis of the amino sugars glucosamine (GlcN) and muramic acid (MurN) (Joergensen and Wichern, 2008; Potthoff et al., 2008). In soils, the fungal cell membrane component ergosterol is a better indicator for fungal biomass, as it does not accumulate in soil organic matter (Joergensen and Wichern, 2008).

The current soil temperature manipulation experiment with litterbags containing maize leaves addresses the following questions: (1) How strong is the infestation and survival of fungal plant pathogens on inoculated maize leaves under increased winter soil temperatures? (2) Is the fungal plant pathogen load related to the fungal and bacterial biomass colonizing maize leaves during decomposition? (3) Do increasing winter soil temperatures and inoculum loads of fungal plant pathogens affect soil microorganisms below the litterbags?

## 2. Material and methods

### 2.1. Soil warming facility

The experimental site is located in the northern part of Göttingen, Lower Saxony, Germany (51°33'29.28"N, 9°55'59.46"E) at 180 m ASL. The mean annual temperature of Göttingen is 8.7 °C and the mean annual precipitation is 644 mm. Exact information on precipitation during the experimental period was given by Siebold and von Tiedemann (2013) as Supporting information. Briefly, the soil warming facility with a total size of 60 m<sup>2</sup> was designed in 2009 (Siebold and von Tiedemann, 2012b), where the original stony and heterogeneous soil was replaced to a depth of 1 m with an arable top soil, i.e. a silty clay loam (Tu4, pH-H<sub>2</sub>O 8.3), classified as Calcaric Cambisol (FAO-WRB, 2014).

The experiment had a completely randomized block design with the following three temperature treatments, each replicated four times: (ST1) ambient soil temperature, (ST2) ambient +1.6 °C, and (ST3) ambient +3.2 °C. The experimental site consisted of 12 plots (2 m × 2.5 m each) arranged in two rows. Heating cables were buried at a depth of 10 cm in each plot, also in the control plots, to ensure equivalent physical conditions. The soil warming treatments were applied to simulate warming scenarios for Lower Saxony up to the year 2050 (medium-term) and the year 2100 (long-term), respectively (Werner and Gerstengarbe, 2007).

### 2.2. Experimental procedure

The soil used for the experiment was taken from the upper 10 cm of an arable field near Witzhausen (51°23'N, 9°55'E, Northern Hesse, Germany) in September 2011, sieved and stored at 4 °C for about two weeks. The soil was classified as a Haplic Luvisol (FAO-WRB, 2014) with the following characteristics: 3.3% sand, 83.4% silt, 13.3% clay, a pH (CaCl<sub>2</sub>) of 6.3, 1.4% total C, 0.14% N and a C/N ratio of 10. Polypropylene cylinders (10 × 10 cm), closed at the bottom with polyethylene mesh (1 mm mesh size), were filled with moist soil equivalent to 350 g dry soil and transferred to the experimental site at the

beginning of October 2011. Per plot, two cylinders were inserted to a depth of 5 cm (24 cylinders in total).

Green maize leaves (*Zea mays* L.) were dried (60 °C), chopped at < 2 cm and stored in a paper bag at 40 °C until the beginning of the experiment. The maize leaves contained 41.2% C and 2.4% N. Macroconidia of *Fusarium culmorum* DSM 62184 (isolated from mouldy maize grain in Germany by E. Seemüller) and *Fusarium graminearum* 210 (isolated from a wheat ear in Göttingen, Germany) and a mixture of mycelium and sclerotia of *Rhizoctonia solani* AG2-2 were used for inoculation. Macroconidia were obtained as described by Becher et al. (2010). Briefly, 50 mL of mung bean (*Vigna radiata* (L.) R. Wilczek) broth was inoculated with potato dextrose agar (PDA) plugs, colonised with *F. graminearum* and *F. culmorum*. These plugs were filled into 300-mL flasks and incubated for 7 days at ambient light and temperature while shaking at 50 rev min<sup>-1</sup>. PDA medium in a single Petri dish was inoculated with an *R. solani* AG2-2 strain and incubated for 4 weeks at room temperature without light. Agar with *R. solani* mycelium was homogenised in a blender and the slurry was transferred into nutrient solution. The inoculum solutions were centrifuged at 4500g for 10 min in 120 mL polypropylene containers to rule out possible effects of the mung bean broth and the sclerotia nutrient solution during the decomposition of maize leaves. The supernatant was discarded carefully and the conidia/sclerotia pellets were dissolved in autoclaved water. This step was repeated until colourlessness of the suspension indicated complete removal of the nutrient solution. The stock solutions were then kept at 4 °C.

Polyethylene litterbags (LB) (8 × 5 cm; 1 mm mesh) were filled with 3 g of the oven-dried maize leaves, closed with staples and placed in a desiccator with moist paper towels for 24 h to reduce water repellence of maize leaves. After remoistening, the maize leaves of 12 litterbags were inoculated at the same time by pipetting 2 mL of each inoculum solution containing 30,000 macroconidia of *F. culmorum* and *F. graminearum*, respectively, and 133.7 mg mycelium/sclerotia of *R. solani*. After inoculation at the end of October, the litterbags were immediately brought to the experimental site together with another 12 litterbags with non-inoculated maize leaves, which received 6 mL autoclaved water and served as controls. Each of the two cylinders per plot received one pathogen-inoculated or one non-inoculated litterbag, placed on top of the soil. Then, the cylinders were covered with a 2 mm polyethylene mesh to protect the litterbags, but allowing precipitation to pass.

At the end of the experiment, litterbags and soil-filled cylinders were recovered. Soil loosely adhering to the litterbags was removed carefully with a brush and a knife prior to opening the bag itself. The maize residues of each litterbag were dried at 40 °C for 48 h, weighed and milled for further analysis.

### 2.3. Microbial biomass indices

Soil microbial biomass C and N were estimated by fumigation extraction (Brookes et al., 1985; Vance et al., 1987). A sub-sample of 20 g moist soil was separated into two portions of 10 g. One portion was fumigated at 25 °C with ethanol-free CHCl<sub>3</sub>, which was removed after 24 h. Fumigated and non-fumigated samples were extracted for 30 min with 40 mL of 0.5 M K<sub>2</sub>SO<sub>4</sub> by horizontal shaking at 200 rev min<sup>-1</sup> and filtered (hw3, Sartorius Stedim Biotech, Göttingen, Germany). Organic C and total N in the extracts were measured using a multi N/C 2100S automatic analyser (Analytik Jena, Germany). Microbial biomass C was calculated as  $E_C/k_{EC}$ , where  $E_C$  = (organic C extracted from fumigated soil) – (organic C extracted from non-fumigated soil) and  $k_{EC}$  = 0.45 (Wu et al., 1990). Microbial biomass N was calculated as  $E_N/k_{EN}$ , where  $E_N$  = (total N extracted from fumigated soil) – (total N extracted from non-fumigated soil) and  $k_{EN}$  = 0.54 (Brookes et al., 1985).

Ergosterol was extracted from 2 g moist soil with 100 mL ethanol (96%) according to Djajakirana et al. (1996). Ergosterol was determined by reversed-phase HPLC analysis (Gynkotek 480, Germering,

Germany; Spherisorb ODS II separation column, Phenomenex C18 ODS guard column) at 26 °C with 100% methanol as the mobile phase and detected at 282 nm (Dionex UVD 170 L, Germering).

## 2.4. Amino sugars

GlcN, galactosamine (GalN), and MurN were determined according to Appuhn et al. (2004) as described by Indorf et al. (2011) using OPA (o-phthalaldehyde) derivatisation. 800 mg of oven-dried (40 °C) maize residue powder were hydrolysed with 10 mL of 6 M HCl for 3 h at 105 °C. Chromatographic separations were performed on a Hyperclone C<sub>18</sub> column (125 mm length × 4 mm diameter) at 35 °C, using a Dionex P 580 gradient pump, a Dionex Ultimate WPS – 3000TSL analytical autosampler with in-line split-loop injection and thermostat and a Dionex RF 2000 fluorescence detector set at 445 nm emission and 330 nm excitation wavelengths. Fungal C was calculated by subtracting bacterial GlcN from total GlcN calculated from MurN, assuming that MurN and bacterial GlcN occur at a 1 to 2 molar ratio in bacterial cells (Engelking et al., 2007):  $\text{mmol fungal C g}^{-1} \text{ dry weight} = (\text{mmol GlcN} - 2 \times \text{mmol MurN}) \times 9$ . Bacterial C was calculated by multiplying the concentration of MurN by 45 (Appuhn and Joergensen, 2006). Microbial C was the sum of fungal C and bacterial C.

## 2.5. DNA extraction and qPCR

DNA extraction from the maize residues was conducted with some modifications as described by Brandfass and Karlovsky (2006) and Becher et al. (2010). Briefly, 50 mg of maize residue powder were blended in a 2-mL tube with 1 mL of cetyltrimethyl-ammonium bromide (CTAB) extraction buffer (10 mM Tris, 20 mM EDTA, 0.02 M C-TAB, 0.8 M NaCl, 0.03 M N-laurylsarcosine, 0.13 M sorbitol, 1% (w/v) polyvinyl-pyrrolidone, pH set to 8.0 with NaOH), 2 µL mercaptoethanol and 1 µL proteinase K (from a stock solution of 20 mg mL<sup>-1</sup>). After an initial incubation for 10 min at 42 °C and a second incubation for 10 min at 65 °C, accompanied by mixing the tubes every 3 min, 0.8 mL of chloroform/isoamyl alcohol (24/1) were added.

The samples were then thoroughly emulsified, incubated on ice for 15 min and centrifuged at 8,000g for 10 min at room temperature. 600 µL of the upper phase were transferred to a 1.5-mL tube containing 200 µL of 30% (v/v) PEG 6000 and 100 µL of 5 M NaCl, mixed and centrifuged at 15,000g for 15 min at room temperature. After carefully decanting the supernatant, the pellets were washed twice with 600 µL 70% (v/v) ethanol, dried, and dissolved in 50 µL of Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH set to 8.0 with HCl). Subsequent analysis of quality and concentration of total DNA was performed by agarose electrophoresis. A 1/20 dilution was used in the PCR. The extraction efficacy of DNA from this particular matrix was not tested, because only negligible amounts of DNA can be recovered from the residue after extracting 50 mg plant material per mL CTAB buffer (Rollwage and Karlovsky, unpublished results).

Quantification of the genomic DNA of *F. culmorum* and *F. graminearum* was carried out by real-time PCR using published protocols (Brandfass and Karlovsky, 2008). For *F. culmorum* the forward (GATGCCAGACCAAGACGAAG) and reverse (GATGCC AGACGCACTAAGAT) primer OPT18 (Schilling et al., 1996) and for *F. graminearum* the forward (ACAGATGACAAGATTCAGGCACA) and reverse (TTCTTTGACA TCTGTTCAACCCA) primer Fg16N (Nicholson et al., 1998) amplified species-specific sequences from the genomes of both species. The amplification mix for *F. culmorum*-specific PCR consisted of NH<sub>4</sub>-reaction buffer (16 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 67 mM Tris-HCl, 0.01% (v/v) Tween-20, pH 8.8 at 25 °C; Bioline, Luckenwalde, Germany), 4 mM MgCl<sub>2</sub>, 0.2 mM of each dATP, dTTP, dCTP and dGTP (Bioline, Luckenwalde, Germany), 0.3 µM of primer OPT18 F and OPT18 R, 0.25 u BIOTaq DNA polymerase (Bioline, Luckenwalde, Germany), 10 nM fluorescein (BioRad, Hercules, CA, USA; to facilitate the collection of well factors), 0. x SYBR Green I solution (Invitrogen, Karlsruhe, Germany), template DNA (1 µL)

and doubly distilled water (ddH<sub>2</sub>O) filled to a total volume of 25 µL.

The amplification mix for the *F. graminearum*-specific PCR consisted of 1 x SYBR Premix Ex Taq (containing TaKaRa Ex Taq HS, dNTP Mixture, Mg<sup>2+</sup>, and SYBR Green I, Takara Bio, Otsu, Japan), 0.3 µM of primer Fg16N F and Fg16N R, 10 nM fluorescein, template DNA (1 µL) and ddH<sub>2</sub>O filled to 25 µL. The iCycler System (BioRad, Hercules, CA, USA) was used for amplification and melting curve analysis with the following temperature profile: initial denaturation for 1.5 min at 95 °C followed by 35 cycles with 30 s at 94 °C, 45 s at 64 °C and 45 s at 72 °C. The final elongation was performed for 5 min at 72 °C. An assay for *R. solani* AG2-2 DNA was used (Abbas et al., 2014), based on a forward primer AG22sp2 (TAGCTGGATCCATTAGTTTG) (Salazar et al., 2000) and reverse primer KhotR (GTTCAAAGAYTCGATGATTAC) (Fredricks et al., 2010), which amplified part of the internal transcribed spacer between ribosomal RNA genes 18S and 5.8S. The qPCR system with SYBR Green detection was optimised for 25 µL qPCR reaction mixture containing PCR buffer (Bioline, Luckenwalde, Germany), 3 mM MgCl<sub>2</sub> (Bioline), 200 µM dNTPs (Bioline), 0.3 µM forward primer, 0.3 µM reverse primers each, 0.1 x of SYBR Green I solution (Invitrogen, Karlsruhe, Germany), 0.25 u of Taq DNA polymerase (Bioline) and 1 µL *R. solani* DNA dilution series from 1.8 pg to 444.4 pg.

The iCycler System (BioRad, Hercules, CA, USA) was used for amplification and melting curve analysis with the following temperature profile: initial denaturation for 3 min at 94 °C followed by 40 cycles with 30 s at 94 °C, 20 s at 59 °C and 30 s at 72 °C. The final elongation was performed for 5 min at 72 °C. Standards were prepared by 3-fold serial dilution from pure genomic DNA of *F. graminearum*, *F. culmorum* and *R. solani*, which was quantified by densitometry as described by Brandfass and Karlovsky (2008) and Abbas et al. (2014). The concentration of fungal DNA determined by qPCR was used to calculate the amount of DNA in maize residues, assuming quantitative DNA extraction.

## 2.6. Statistical analysis

The data presented in tables and figures are arithmetic means expressed on an oven-dry basis (about 24 h at 105 °C). Normality of data distribution was tested using the Kolmogorov-Smirnov and Shapiro-Wilk test and data were transformed when appropriate. To test for treatment effects, a two-way analysis of variance was performed using a general linear model and inoculation treatments (non-inoculated, inoculated) as well as mean soil temperatures of the ST1, ST2 and ST3 plots as fixed factors. The significance of differences between the soil temperature treatments was tested by one-way analysis of variance (ANOVA) using post hoc Tukey HSD ( $P \leq 0.05$ ). All statistical calculations were performed by SPSS Statistics 17.0 (SPSS Inc., Chicago, USA).

## 3. Results

### 3.1. Effects of soil heating on soil temperature and water content

From the end of October 2011 until the end of March 2012, differences in mean daily temperatures at 5 cm soil depth were 1.3 °C ( $\pm 0.5$ ) and 2.7 °C ( $\pm 0.4$ ) between the unheated ST1 plots and the ST2 and ST3 plots, respectively (Fig. 1). Over the 152 days, mean soil temperature was 5.0 °C in the ST1 plots, 6.4 °C in the ST2 plots and 7.7 °C in the ST3 plots. Over the course of the field trial, 15 days with soil temperatures below 0 °C were recorded in the ST1 plots at 5 cm depth. Both soil warming treatments reduced the number of days with soil frost to 11 and 6 in the ST2 and ST3 plots, respectively. Additionally, heating of the surrounding soil significantly decreased the soil water content in the cylinders from 22% in the ST1 plots to an average of 16% in the ST2 and ST3 plots ( $P < 0.05$ ).

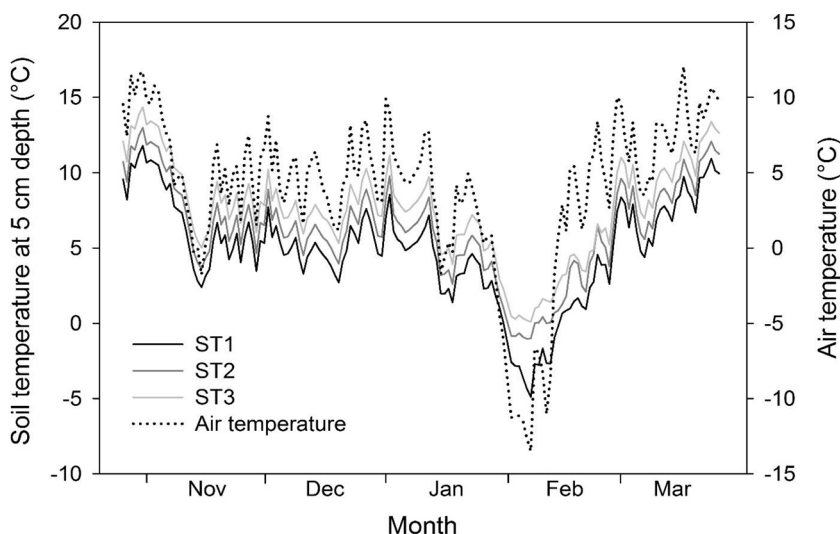


Fig. 1. Temperatures at 5 cm soil depth in plots of the ST1 (ambient soil temperature) and the two soil warming treatments (ST2: ambient + 1.6 °C and ST3: ambient + 3.2 °C) as well as the air temperature based on daily mean temperatures.

### 3.2. Soil microbial biomass indices

The contents of soil microbial biomass C and biomass N at the end of the experimental period were very similar between all inoculation and soil temperature treatments and averaged 301  $\mu\text{g}$  and 58.5  $\mu\text{g g}^{-1}$  soil, respectively (Table 1). The average soil microbial biomass C/N ratio was 5.4. All samples contained around 0.33  $\mu\text{g ergosterol g}^{-1}$  soil and had an average proportion of ergosterol to microbial biomass C of about 0.12%. For all soil microbial biomass indices, no significant effects of mean soil temperature, inoculation or temperature  $\times$  inoculation interaction were found (two-way ANOVA).

### 3.3. Decomposition of maize leaves

In the non-inoculated and inoculated litterbags, around 52% and 57% of the maize leaves were decomposed, respectively. A significantly stronger decomposition of about 9% was observed for pathogen-inoculated litterbags of the ST3 plots when compared with inoculated litterbags of the unheated ST1 plots. In addition, a significant 10% increase in decomposition over the respective non-inoculated maize leaves was found for the pathogen-inoculated maize leaves of the ST2 and ST3 plots ( $P < 0.05$ ). Regardless of the soil heating treatments, this resulted in a maize decomposition rate of about 10.3  $\text{mg DW d}^{-1}$  in non-inoculated and 11.3  $\text{mg DW d}^{-1}$  in pathogen-inoculated litterbags.

Table 1

Contents of microbial biomass C and N, biomass C/N ratio, ergosterol and ergosterol to biomass C at the end of the 152-day field experiment in the soil below the non-inoculated and inoculated litterbag samples of all temperature treatments; none of the treatments and interactions was significant (two-way ANOVA,  $P > 0.05$ ,  $n = 24$ ).

	Microbial biomass			Ergosterol ( $\mu\text{g g}^{-1}$ soil)	Ergosterol/ biomass C (%)
	C	N	C/N		
	( $\mu\text{g g}^{-1}$ soil)				
Non-inoculated					
ST1	272	48	5.8	0.32	0.12
ST2	293	82	4.6	0.35	0.14
ST3	292	57	5.2	0.31	0.11
Inoculated					
ST1	320	56	5.8	0.31	0.10
ST2	314	54	5.7	0.36	0.12
ST3	315	54	5.5	0.31	0.10
CV ( $\pm$ % )	14	21	13	20	19

ST1: ambient soil temperature; ST2: ambient soil temperature + 1.6 °C; ST3: ambient soil temperature + 3.2 °C; CV = pooled coefficient of variation between replicates ( $n = 4$ ).

Only within the ST2 and ST3 plots was the decomposition rate of the pathogen-inoculated maize leaves significantly increased, by about 1  $\text{mg d}^{-1}$  over the non-inoculated litterbags ( $P < 0.05$ ). Mean soil temperature ( $P < 0.05$ ) and pathogen inoculation ( $P < 0.001$ ) had significant effects on maize residue decomposition (two-way ANOVA).

### 3.4. Maize-colonizing bacterial and fungal biomass

Total amino sugar contents increased from 1920 (ST1) to 3030 (ST2 and ST3)  $\mu\text{g g}^{-1}$  DW on non-inoculated maize leaves and varied around 2110  $\mu\text{g g}^{-1}$  DW in pathogen-inoculated litterbags (Table 2). Significant treatment effects were found for mean soil temperature and pathogen inoculation (two-way ANOVA,  $P < 0.01$ ). The highest amino sugar contents found on the non-inoculated maize leaves of the ST2 and ST3 plots were on average 36% higher than in the respective pathogen-inoculated litterbags. GlcN was the most abundant amino sugar, on average accounting for 87% in all treatments. Highest GlcN and fungal C contents were found again in the non-inoculated litterbags of the ST2 and ST3 plots. Here, the values on maize leaves of the ST3 plots were increased by about 47% over the respective pathogen-inoculated litterbags ( $P < 0.05$ ). GlcN and fungal C were significantly affected by mean soil temperature, inoculation treatment as well as their interaction (two-way ANOVA, Table 2).

MurN accounted for 1.5% of total amino sugars and varied around a mean of 36  $\mu\text{g g}^{-1}$  DW, resulting in 1.64  $\text{mg bacterial C g}^{-1}$  DW, without significant treatment effects (two-way ANOVA). However, one-way ANOVA within pathogen-inoculated maize leaves revealed a significant increase of about 37% for the ST2 and ST3 plots over samples of the ST1 plots (Tukey HSD,  $P < 0.05$ ). The fungal C to bacterial C ratio varied around 10.4 and significantly increased from 9.7 (ST1) to 17.2 (ST3) on maize leaves without pathogen inoculation. Again, the fungal C to bacterial C ratio was significantly wider in the non-inoculated litterbags of the ST3 plots than in the respective pathogen-inoculated litterbags ( $P < 0.05$ , Table 2).

On average, GalN contributed 10% to total amino sugars on non-inoculated and 6% on pathogen-inoculated maize leaves. In both inoculation treatments, soil warming did not affect GalN. However, the GalN content in pathogen-inoculated maize leaves was on average 47% lower than that in non-inoculated leaves, leading to a significant inoculation effect (two-way ANOVA,  $P < 0.01$ ).

### 3.5. Fungal plant pathogens

At the beginning of the experiment, the target species were almost not present on the non-inoculated plant material. The fresh and green

**Table 2**

Concentrations of total amino sugars, muramic acid (MurN), glucosamine (GlcN), galactosamine (GalN); concentrations of fungal C and bacterial C, calculated from fungal GlcN and MurN as well as the fungal C to bacterial C ratio in inoculated and non-inoculated maize leaves of all temperature treatments at the end of the 152-day field experiment.

Treatment	Total amino sugars ( $\mu\text{g g}^{-1}$ DW)	GalN	GlcN	MurN	Fungal C ( $\text{mg g}^{-1}$ DW)	Bacterial C	Fungal C/bacterial C
Non-inoculated							
ST1	1922 b	259 a	1582 b	34.5 a	13.6 b	1.6 a	9.7 b
ST2	2953 a	246 a	2580 a	54.4 a	22.7 a	2.4 a	11.5 ab
ST3	3109 a	264 a	2715 a	31.7 a	23.8 a	1.4 a	17.2 a
Inoculated							
ST1	1854 a	120 a	1609 a	26.1 b	14.0 a	1.2 b	12.0 a
ST2	2378 a	134 a	2126 a	35.1 a	18.5 a	1.6 a	11.7 a
ST3	2103 a	151 a	1842 a	36.6 a	15.9 a	1.6 a	9.7 a
Probability values							
Temperature	0.01	NS	< 0.01	NS	< 0.01	NS	NS
Inoculation	0.01	< 0.01	0.01	NS	0.01	NS	NS
T $\times$ I	NS	NS	0.03	NS	0.02	NS	0.03
CV ( $\pm$ %)	12	27	12	23	12	23	23

ST1: ambient soil temperature; ST2: ambient soil temperature + 1.6 °C; ST3: ambient soil temperature + 3.2 °C; values within a column of an inoculation treatment followed by the same letter are not significantly different at  $P \leq 0.05$ ; NS = not significant; CV = pooled coefficient of variation between replicates ( $n = 4$ ).

maize leaves were only slightly colonised by fungi, as the leaves had no contact to soil organisms during growth. At the end of the experiment, inoculated maize leaves were dominated by *F. culmorum* in all temperature treatments (95% of measured pathogen DNA (ST1), 98% (ST2), and 91% (ST3)). The amount of DNA applied with macroconidia increased from 0.49 ng mg<sup>-1</sup> DW to an average of 2390 ng mg<sup>-1</sup> DW after 152 days, without significant differences between soil heating levels (Fig. 2a). However, in both soil-warming treatments, *F. culmorum* DNA on maize leaves decreased by about 13% in comparison with the non-heated control plots. The amount of *F. graminearum* DNA applied increased from 0.4 ng mg<sup>-1</sup> DW to 15 ng mg<sup>-1</sup> DW at ambient temperature and to 32 ng mg<sup>-1</sup> DW at the highest warming level (Fig. 2b), leading to a strong positive correlation with mean soil temperature ( $r = 0.91$ ,  $P < 0.0001$ ). About 427 ng of *R. solani* DNA mg<sup>-1</sup> DW were applied as a mixture of hyphae and sclerotia (Fig. 2c). This content decreased between 93% (ST2) and 46% (ST3).

## 4. Discussion

### 4.1. General temperature effects on decomposition and microorganisms

After 152 days, 57% of maize leaves were decomposed in the litterbags under field conditions, without consistent soil warming effects. The resulting average decomposition rate constant  $k$  of 0.0016 d<sup>-1</sup> ( $C(t) = C(0) e^{-kt}$ ) was in the range of  $k = 0.0074$  at 8.4 °C reported for maize leaves (Jacobs et al., 2011) or  $k = 0.0011$  d<sup>-1</sup> at 11.7 °C (Mungai and Motavalli, 2006) or  $k = 0.0039$  d<sup>-1</sup> at 6 °C (Burgess et al., 2002) for maize leaf straw in litterbags. Soil microorganisms below the litterbags were not affected by increasing winter soil temperatures for 152 days. This is in accordance with Schindlbacher et al. (2011), who observed no effects on microbial biomass C and biomass N by a 4.0 °C elevation in temperature for 4 to 6 years. In contrast, Grunwald et al. (2017) observed a significant increase in microbial biomass C by a 2.5 °C elevation in temperature for 5 years for unknown reasons. In non-inoculated litterbags, elevated soil temperatures did not affect decomposition and the maize leaf colonizing bacterial biomass but increased the saprotrophic fungal biomass. In pathogen inoculated litterbags, elevated soil temperatures significantly increased decomposition in comparison with the ambient temperature plots and with both warming treatments of the non-inoculated litterbags. Due to the decomposition of maize leaves, the increase in DNA of *Fusarium* species per litterbag was less strong than indicated by the concentration per DW, whereas the decrease in *R. solani* DNA was even stronger. Soil

microorganisms below the inoculated litterbags were also not affected by the load of fungal plant pathogens, which might have reached the soil by hyphal growth or by rainfall leaching.

Amino sugar concentrations showed a low initial microbial colonisation of non-inoculated green maize leaves in agreement with Indorf et al. (2011), contrasting those reported by Rottmann et al. (2011) for mature maize straw. MurN was not detectable on the original material, indicating the absence of bacterial colonisation of green plant leaves. At the end of the field experiment, total amino sugars on both pathogen-inoculated and non-inoculated maize leaves generally had very large proportions of fungal GlcN (on average 84%), which were very similar between all treatments. Fungi dominated microbial colonisation of plant residues in comparison with bacteria (Schneider et al., 2012), especially in winter, as reported by Lipson et al. (2002) and Schadt et al. (2003).

In the present experiment, mean soil temperature was significantly correlated with fungal GlcN ( $r = 0.74$ ,  $P < 0.01$ ) but not with MurN in non-inoculated maize leaves. In contrast, mean soil temperature was correlated with MurN ( $r = 0.84$ ,  $P < 0.01$ ) but not with fungal GlcN in the pathogen-inoculated maize leaves. This suggests that the presence of *Fusarium* species and *R. solani* inoculants disturbed the temperature-dependent development of maize-leaves colonizing saprotrophic fungi. However, these relationships should not be over-interpreted, considering the strong variation in GlcN and MurN concentrations. The mean temperature-specific coefficients of variation varied around 29% on the non-inoculated and around 7% on the pathogen-inoculated maize leaves. These large variations are caused by highly variable interactions between bacteria, saprotrophic and pathogenic fungi, which may mask existing temperature effects.

### 4.2. Fungal plant pathogen inoculum

Among the inoculated plant pathogens, *F. culmorum* was most dominant and showed the greatest growth potential on aboveground maize leaves during winter. This is similar to previous results of Lukas et al. (2014), investigating the survival of different plant pathogens on maize leaves at temperatures around freezing point in a laboratory experiment. In the current study, the differences between the ST1 plots and the soil heating treatments were not significant, which might be due to the limited number of replicates per warming treatment and the resulting high variation between plots. Residue decomposition was not correlated with the amount of pathogen DNA at the end of the experiment, suggesting that an increased microbial saprotrophic activity

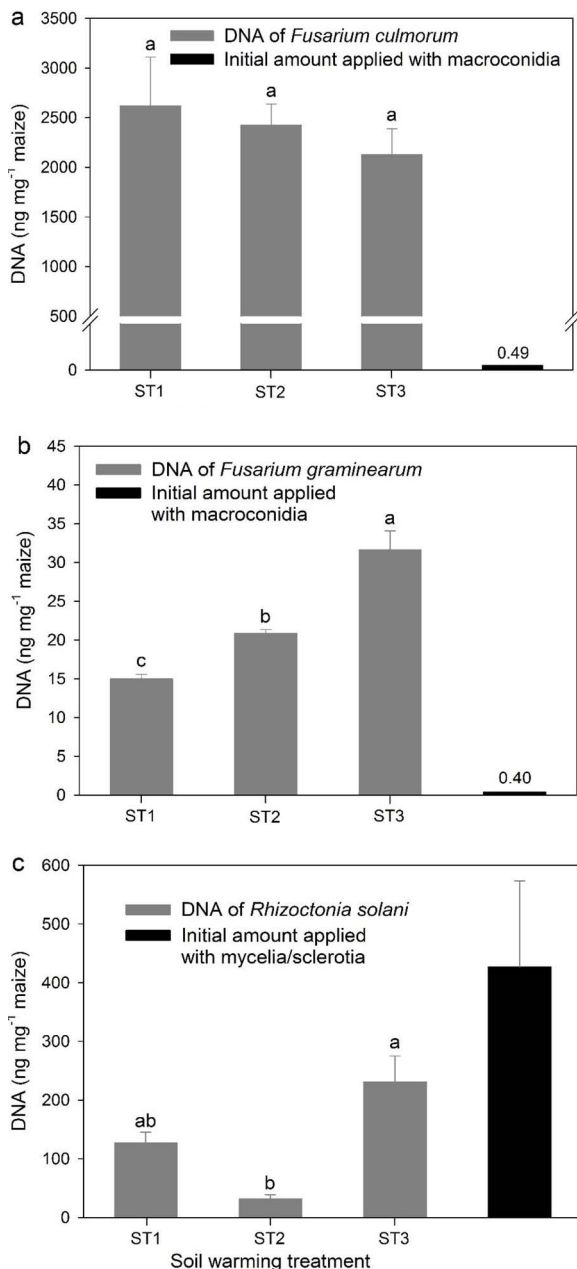


Fig. 2. Contents of DNA of (a) *Fusarium culmorum*, (b) *Fusarium graminearum* and (c) *Rhizoctonia solani* of the inoculated maize leaves in the ST1 (ambient soil temperature) and the two soil warming treatments (ST2: ambient + 1.6 °C and ST3: ambient + 3.2 °C) at the end of the 152-day field experiment; error bars show + one standard error of mean; different letters above the columns indicate significant differences ( $P < 0.05$ ).

due to future soil warming in winter will not necessarily be related to an increased abundance of pathogenic fungi on crop residues.

Furthermore, Bowen (1990) reported a rapid decomposition of wheat residues inoculated with *F. culmorum* during the first three weeks, which gradually declined thereafter. It is known that *F. culmorum* can degrade cellulose and hemicelluloses (Harper and Lynch, 1985) but is not able to degrade lignin (Bowen and Harper, 1988; Deacon et al., 2006). After the depletion of cellulose and hemicelluloses, *Fusarium* species seem to be replaced by other microorganisms, further degrading the residues (Bowen, 1990; Palazzini et al., 2013). This would also explain the lower colonisation in the later stage of substrate decomposition at the end of the experiment, when easily available nutrients were exhausted.

In the current experiment, the large amounts of pathogen DNA as

well as the temperature-independent extended survival point to an elevated disease potential for agricultural crops susceptible to *F. culmorum* at increased winter temperatures in the future (Juroszek and von Tiedemann, 2015; Newbery et al., 2016). However, a single-season experiment is certainly not sufficient for exact prognosis of future trends in pathogen development, especially considering several experimental drawbacks of our approach: Cultured pathogens certainly behave differently to those surviving in soil under true field conditions. A soil warming system close to a building (Siebold and von Tiedemann, 2012b, 2013), but also litterbags (Knacker et al., 2003) have strong microclimatic effects, changing survival and saprotrophic activities of fungi. The same is true for the soil type, where sandy or clayey soils differ in their habitat properties from the current loam.

In contrast to *F. culmorum*, *F. graminearum* is most common in warm and continental climates (Nielsen et al., 2011; Parikka et al., 2012) and the germination of conidia as well as fungal growth is favoured by warm and humid conditions (Leplat et al., 2013). The optimum temperature for mycelial growth is 25 °C, whereas no growth was observed at temperatures below 5 °C (Ramirez et al., 2006). In comparison with *F. culmorum*, the results of our study revealed a considerably lower ability of *F. graminearum* to colonize maize leaves under field conditions in winter, underlining its poor adaptation to low temperatures. It is also known that *F. graminearum* is a poor competitor for the colonisation and decomposition of crop residues over time, especially in contact with soil (Leplat et al., 2013, 2016). *F. graminearum* is often rapidly replaced from colonised residues by other competitors including other *Fusarium* species (Palazzini et al., 2013; Pereyra et al., 2004). However, in our experiment no correlation between the biomass of *F. graminearum* and *F. culmorum* was found.

In comparison with the initial amount of DNA, growth of *Fusarium* species was generally found during winter in all treatments. However, it should be considered that some DNA might be extracted from dead fungal residues (Carini et al., 2016). DNA concentration of *F. graminearum* significantly increased with elevation of mean soil temperature, despite temperatures considerably below optimum, whereas that of *F. culmorum* showed a tendency to decline. This suggests that accelerated substrate decomposition in future warmer winters may reduce the dominance of one pathogen, e.g. *F. culmorum*, and may simultaneously improve conditions for the development of another, e.g. *F. graminearum*. Our results are in line with others, who reported a decline of *F. culmorum* and a recently increased prevalence of *F. graminearum* in the Netherlands (Waalwijk et al., 2003), in Denmark (Nielsen et al., 2011), and in Norway (Hofgaard et al., 2016). However, it remains unknown whether an increased infection risk for *Fusarium* head blight caused by *F. graminearum* as suggested by the simulation study of Volk et al. (2010) for North Rhine-Westphalia (Germany) will really increase the inoculum load at winter's end (Newbery et al., 2016). It should be considered that the disease pressure after winter not only depends on the primary inoculum but also on moisture of harvest residues and weather conditions in the following growing season (Juroszek and von Tiedemann, 2015; Manstretta and Rossi, 2015, 2016).

*R. solani* has some advantages in colonizing organic matter, due to its saprotrophic and biotrophic life modes in comparison with *Fusarium* species. The germination of sclerotia in the soil or on plant residues and their ability to initiate the growth of hyphae depends very much on the temperature (Ritchie et al., 2009). They found an optimal temperature range for sclerotial germination between 15 and 25 °C in soil, whereas germination was completely inhibited at 5 °C. The saprotrophic life-style of *R. solani* is stimulated by increasing decomposition stage, whereas *Fusarium* species may directly infect green plant material. Due to the strong response to environmental factors, sclerotial germination and hyphal growth exhibits strong spatial variability (Boine et al., 2014), often forming hotspots in disease prevalent on arable fields, especially under maize and sugar beet (Kiewnick et al., 2001; Kühn et al., 2009).

DNA concentration of *R. solani* DNA on inoculated maize leaves

after 152 days was greatly reduced compared with the initial inoculation. This suggests that the mixture of mycelia and sclerotia applied as inoculum was partly decomposed by other microorganisms. Another possibility would be that a part of the inoculum, especially the large sclerotia were simply washed away during the experimental period. In line with this assumption, the highest amount of GlcN was observed in the soil warming treatment with the lowest amount of *R. solani* DNA. It is also possible that fungi other than the *Fusarium* spp. used in this study grew at the expense of *R. solani*. Mycoparasites (e.g. *Verticillium biguttatum* and *Trichoderma virens*) are known to destroy sclerotia of *R. solani* (Demirci et al., 2009; Liu et al., 2010). *Trichoderma* species are able to hydrolyse fungal structures (e.g. conidia and mycelia) via the production of various enzymes (Cooney et al., 2001) and utilize fungal mycelium as a nutrient source (Popiel et al., 2008). Ritchie et al. (2013) reported that only 60% of buried sclerotia of *R. solani* could be retrieved viable after 18 months. They also observed a strong decrease in sclerotia viability (between 10% and 35% remained viable). In our study, based on quantification of DNA, between 7% and 54% of the applied sclerotia were found at the soil surface after five months. This indicates an increased degradation when sclerotia are directly exposed to the environment.

## 5. Conclusions

On pathogen-inoculated maize leaves, *F. culmorum* DNA revealed the strongest increase during winter, *F. graminearum* a considerably smaller one, whereas *R. solani* DNA declined in comparison with the initial values. This was partly due to microbial decomposition and partly due to wash-out by rainfall. Only *F. graminearum* DNA significantly increased with rising winter soil temperatures of 1.3 and 2.7 °C above ambient soil temperature, which is far removed from the optimum growth temperature for fungal plant pathogens. Rising winter soil temperatures will most likely cause shifts within the plant residue colonizing fungal community, especially between different *Fusarium* species. Maize leaf decomposition was increased by soil warming after inoculation with fungal plant pathogens. However, this increase did not significantly reduce the pathogen load and was also not reflected by total fungal or bacterial biomass. In contrast, soil warming increased total fungal biomass on non-inoculated maize leaves, but did not accelerate their decomposition. Soil microorganisms below the litterbags were affected neither by increasing winter soil temperatures nor by the inoculum loads of fungal plant pathogens. A single-season experiment is probably not sufficient for exact prognosis of future trends in pathogen development in winter periods.

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